Anal. Biochem., 194(1), 25-33 (1991)

Synthesis of Methyl 5-*O-trans*-Feruloyl-α-L-arabinofuranoside and Its Use as a Substrate to Assess Feruloyl Esterase Activity[1]

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A synthetic scheme was developed for the production of methyl 5-*O-trans*-feruloyl-α-L-arabinofuranoside (FA-Ara) in gram quantities. This molecule accurately models the chemical attachment of ferulic acid to polysaccharides found in cell walls of plants in the Gramineae family. It is therefore a realistic substrate that can be used to monitor feruloyl esterase activity. Ultraviolet spectral analysis indicated that FA-Ara has an absorption maximum distinct from the hydrolytic product, ferulic acid (FA), over a wide range of solution pH values. The log molar extinction coefficient ranges from 4.16 to 4.36 for FA-Ara and 4.16 to 4.33 for FA depending upon the pH of the buffered solution. Consequently a convenient spectrophotometric assay can be utilized to monitor esterase activity. Three different methods were developed for using this model substrate to assess esterase activity, including thin-layer chromatography, a spectrophotometric assay, and the use of high-performance liquid chromatography.

Ferulic acid is a cell wall component within plants of several monocot families (1–4), including Gramineae, and the dicot families Solanaceae (5) and Chenopodiaceae (6). This hydroxycinnamic acid is covalently linked to lignin, suberin, and structural polysaccharides (7). Linkage to lignin can be by ester or ether bonds, although the specific components of lignin involved and the regiochemistry have not been determined. Conversely, linkage of ferulic acid to polysaccharides is highly specific. Isolated feruloylated oligosaccharides from Gramineae cell walls consistently showed ferulic acid attached to C-5 of L-arabinofuranosyl side chains on arabinoxylans. The major oligomers include: 3-*O*-(5-*O-trans*-feruloyl-α-L-arabinofuranosyl)-D-xylose (8–10), *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (11), and recently, *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(*trans*-feruloyl-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (12). Reports of detailed structural analysis of feruloylated polysaccharides from dicotyledons are less frequent. Unlike the grasses, dicot ferulic acid is associated with the pectic polysaccharides. Fry isolated two feruloylated oligosaccharides (5), 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose and 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose, that were derived from the pectic fraction of the cell wall. Whether these associations represent the only types of linkages between ferulic acid and polysaccharide components remains to be determined. The consistency and specificity of the linkage pattern, at least in the grass species, may be indicative of similar structural roles.

The roles of wall-bound ferulic acid have not been completely elucidated. Ferulic acid released during saponification of cell walls is correlated with an increase in microbial degradation (13–15) and enzymatic degradation (16) of wall polysaccharides, suggesting structural roles that limit polysaccharide degradation. Isolation of diferulic acid (17) led to the hypothesis that dimers may also cross-link polysaccharides. Fry proposed that polysaccharides cross-linked by diferulic acid may restrict cell wall extensibility (18,19). Recent work with *Avena* seedlings demonstrated a positive correlation between extractable diferulic acid and decreased coleoptile elongation (20). In addition, the bifunctional nature of ferulic acid itself has lead to the

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speculation that ferulic acid may act as a cross-link between specific polysaccharides and lignin (21). Proof for these roles within cell wall matrices awaits further structural analysis.

Elucidation of the structural roles of wall-bound ferulic acids requires detailed analysis of cell-wall components. Enzymatic degradation of cell walls can generate fragments containing ferulic acid that are amenable to structural analysis. Selection of appropriate enzyme preparations requires that they be devoid of feruloyl esterase which could inadvertently remove all or part of the ferulic acid. Some commercial enzyme preparations reportedly contain feruloyl esterase activity (17), whereas others appear to be free of such activity (18). Purified feruloyl esterase could be used to remove ferulic acid from cell walls prior to treatment with polysaccharide hydrolases to assess its impact upon cell wall degradation. It has been proposed that phenolic acid esterases in plant cell walls may be involved in the turnover of wall-bound ferulic and diferulic acid (22). Controlling the spatial arrangement of ferulic acid within the matrix could regulate diferulic acid formation, and, in turn, extensibility. Esterase activity, measured using α-naphthyl acetate as substrate, was identified in corn root epidermal cells corresponding to zones of rapid elongation (22). Further understanding of the relationship between esterase activity and cell elongation will require isolation and characterization of the wall esterases.

Most investigations concerned with the determination of esterase activities utilize artificial substrates such as *p*-nitrophenyl acetate and α-naphthyl acetate, which provide a rapid spectrophotometric method. However, these substrates may not reveal the specificity of esterase activities. If a highly specific esterase such as feruloyl esterase is present, artificial substrates may produce erroneous results. Recent studies have employed methyl (23) and ethyl ferulate (24), as well as cell wall extracts released from "cellulase-treated" plant cell walls (25,26). Methyl and ethyl ferulate provide the proper phenolic residue but lack a realistic alcohol moiety which for most plants is L-arabinose. The procedure for purification of a feruloylated arabinoxylan oligomer provides only small quantities (5 mg from 380 g bermuda grass) (26) of the esterase substrate.

To overcome the limitations of the aforementioned substrates, a simple procedure was designed for the gram-scale synthesis of methyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside (FA-Ara)[2] as an esterase substrate. This paper describes a straightforward method for the synthesis of the substrate, its chemical characteristics, and methods for its use as a substrate in screening and possible enzyme purification procedures. This substrate models the major feruloyl linkage to polysaccharides and provides a material with well-defined chemical properties.

[2] Abbreviations: MeOH, methyl alcohol; ETOH, ethyl alcohol; DTE, dithioerythritol; FA, ferulic acid; FA-Ara, methyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside; DMSO, dimethyl sulfoxide; COSY, correlation spectroscopy.

MATERIALS AND METHODS

General

Evaporations were conducted *in vacuo* at 40°C unless otherwise noted. Melting points are uncorrected. Silica gel chromatography was performed with a standard flash (27) apparatus (Ace Glass) using Kieselgel 60 (Merck). TLC was with Alugram Sil G/UV_{254} plates (Macherey-Nagel). The solvents used were A, chloroform:ethyl acetate (19:1); B, chloroform:ethyl acetate (4:5); C, ethyl acetate; and D, ethyl acetate:acetic acid (100:1). NMR data were determined with a Bruker AM-360 360-MHz (wide-bore) instrument, using the central solvent signal as internal reference (acetone-d_6, ^{1}H, 2.04 ppm, and ^{13}C, 29.8 ppm; DMSO-d_6, ^{1}H, 2.49 ppm, and ^{13}C, 39.5 ppm). One- and two-dimensional NMR spectra were obtained using standard pulse sequence programs, with unambiguous assignments based on COSY, C–H correlation, and long-range C–H correlation spectra. The long-range C–H correlation spectrum, Fig. 2, was run using the microprogram XHCORR.AU with 8K data points in t_2 and 256 zero-filled to 512 data points in t_1. The apodization was simple exponential multiplication in t_2 (LB = 3 Hz) and sine-bell-squared in t_1. Delay times $\Delta 1$ and $\Delta 2$ (28) of 35 and 25 ms were used (29). Ultraviolet spectra were obtained with a Beckman DU-50 spectrophotometer at 500 nm/min; data were collected on a computer interfaced with the spectrophotometer utilizing Datacapture software (Beckman). The synthetic scheme for methyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside is shown in Fig. 1.

Synthesis of Methyl 5-O-trans-Feruloyl-α-L-arabinofuranoside

Carbohydrate moiety. L-Arabinose (**1**, Fig. 1, 10 g, 67 mmol; Sigma) was converted to a crude mixture of predominantly the methyl L-arabinofuranosides, **2**, by treatment with sulfuric acid (1.47 g) in MeOH (160 ml for 24 h) (30). The solution was neutralized with pyridine (30 ml) and evaporated (60°C) to a syrup. The syrup was diluted with pyridine (50 ml) and evaporated again (31).

The mixture was diluted with pyridine (75 ml) and cooled on ice, and benzoyl chloride (31 ml, 4 eq) added with vigorous stirring. A precipitate formed immediately. The mixture was removed from the ice bath after 10 min and left stirring at room temperature for 1 h. Excess benzoyl chloride was quenched with H_2O and the mixture transferred to a separatory funnel. The solution was diluted with methylene chloride and washed sequentially with H_2O, 3 N H_2SO_4, H_2O, and aqueous

$NaHCO_3$ (31). The organic layer was dried over Na_2SO_4, filtered, and evaporated to a thick yellow syrup. Two crystallizations from 95% ethanol yielded methyl 2,3,5-tri-*O*-benzoyl-α-L-arabinofuranoside (**3**) as long (colorless) needles (9.83 g, 31%, mp 103–105°C; R_f 0.67 in solvent A). Difficulty was encountered in trying to scale-up this reaction. Thus, several preparations provided a large quantity of **3**.

The tribenzoate **3** (13.45 g, 28.22 mmol) was suspended in MeOH (300 ml) and stirred overnight in the presence of sodium methoxide (180 mg, Fluka). The mixture was neutralized with Amberlite IR-120 (H^+) resin and taken to a syrup. Three additions and evaporations (50°C) of water eliminated the remaining benzoic acid. The resulting syrup was diluted with ethyl acetate and dried over Na_2SO_4. Crystallization from ethyl acetate generated methyl α-L-arabinofuranoside (**4**) as extremely hygroscopic white needles which were stored over P_2O_5 (3.91 g, 84%; R_f 0.18 in solvent C).

Phenolic acid moiety. Ferulic acid (**5**, Sigma, 20 g, 103 mmol) was acetylated with acetic anhydride (32 ml, 339 mmol) in pyridine (35 ml). The reaction was quenched after 4 h with 95% EtOH (25 ml). Upon cooling, the solution deposited crystalline 4-acetoxyferulic acid (**6**, 17.44 g). The remaining filtrate was taken to a syrup, diluted with toluene, and evaporated to a syrup. The remaining pyridine was eliminated by a second co-distillation with toluene. Redissolution of the resulting syrup with 95% EtOH yielded a second crop of crystals (6.16 g). Recrystallization of the combined extracts from 95% EtOH gave pure **6** (21.57 g, 88.6%; mp 201–204°C, R_f 0.58 in solvent D).

Crystalline **6** (4.04 g, 17 mmol) was placed in a round-bottomed flask (200 ml) along with a stir bar, benzene (75 ml), and thionyl chloride (5 ml, 68.5 mmol). The solution was placed in an oil bath (95°C) and refluxed for 40 min. The mixture was then evaporated under reduced pressure to a solid, redissolved in toluene, and evaporated again. The resulting material was dissolved in hot toluene and, upon cooling, provided 4-acetoxyferuloyl chloride (**7**) as small white plates (3.18 g, 73.6%; mp 130–133°C) that were stored *in vacuo* over P_2O_5.

Coupling and deprotection. The acid chloride **7** (2.17 g, 8.52 mmol) was dissolved in toluene and placed in a dropping funnel under N_2. The solution was added dropwise to a continuously stirring ice-cold mixture of **4** (1.50 g, 9.12 mmol) in pyridine (20 ml). Once the addition was complete (approximately 30 min), the mixture was left unstirred for 4 h. The resulting mixture was taken to a syrup and twice diluted with toluene and evaporated. The solid reaction product was purified by silica gel chromatography (70 g, solvent A) to yield **8**, which crystallized as small white needles from the syrup (1.813 g, 56%; mp 107–110°C, R_f 0.45 in solvent C).

The removal of the phenolic hydroxyl acetate protecting group was achieved with piperidine in 95% ethanol. Crystalline **8** (610 mg, 1.6 mmol) was dissolved in 95% ethanol (25 ml) under N_2. Piperidine (32 μl, 3.2 mmol) was added, whereupon the solution turned yellow. The solution was left for 30 min and quenched with acetic acid (40 μl). The mixture was taken to a syrup and subjected to silica gel chromatography (40 g, solvent B) to provide **9** as a syrup (525 mg, 96%). The final product (**9**) was crystallized from methylene chloride as off-white needles (367 mg, 67%; mp 70–72°C, R_f 0.39 in solvent C). ^1H NMR: δ 3.32 (3 H, s, OCH_3), 3.90 (3 H, s, Ar-OCH_3), 3.95 (1 H, dt, $J_{2',3'}$ = 3.7 Hz, $J_{3',4'} + J_{3',OH}$ = 12.4 Hz, H-3′), 4.04–4.07 (1 H, m, H-2′), 4.13 (1 H, dt, $J_{4',5'b}$ = 3.5 Hz, $J_{3',4'} + J_{4',5'a}$ = 12.6 Hz, H-4′), 4.27 (1 H, dd, $J_{4',5'a}$ = 6.3 Hz, $J_{5'a,5'b}$ = 11.8 Hz, H-5′a), 4.40 (1 H, dd, H-5′b), 4.44 (0.8 H, d, $J_{3',OH}$ = 6.3 Hz, OH-3′), 4.64 (0.8 H, d, $J_{2',OH}$ = 4.7 Hz, OH-2′), 4.80 (1 H, d, $J_{1',2'}$ = 1.7 Hz, H-1′), 6.41 (1 H, d, $J_{7,8}$ = 15.9 Hz, H-8), 6.86 (1 H, d, $J_{5,6}$ = 8.2 Hz, H-5), 7.12 (1 H, dd, $J_{2,6}$ = 2.0 Hz, H-6), 7.31 (1 H, d, H-2), 7.62 (1 H, d, H-7).

Properties of methyl 5-O-trans-Feruloyl-α-L-arabinofuranoside (FA-Ara, 9) Important for Use as an Esterase Substrate

Effect of pH on absorption spectrum. Universal buffer solutions with pH values from 5 to 12 in 1 pH unit increments were prepared according to the procedure of Carmody (32). Sodium azide was added to a final concentration of 0.01% and the pH adjusted if necessary with 0.2 M boric acid + 0.05 M citric acid solution or 0.1 M Na_3PO_4 solution. A stock solution of FA-Ara (**9**) was prepared in distilled H_2O (466.6 μg/ml, 1.38 mM). A 40-μl sample of the FA-Ara stock solution was added to 460 μl of universal buffer, mixed, and the absorption spectrum recorded between 500 and 250 nm. Aqueous ferulic acid (FA, **5**) was prepared by initially dissolving FA in methanol (7 mg/ml) and adding 17 μl to 883 μl of H_2O (0.68 mM). The pH effect on absorbance was determined as described for FA-Ara. Molar extinction coefficients were calculated for FA-Ara and FA for the absorption maximum at each pH.

Stability of FA-Ara complex at high pH. A 40-μl sample of FA-Ara stock solution was added to 460 μl of 200 mM Na_2CO_3 (final pH 11.5) and mixed, and the absorption spectrum recorded between 500 and 250 nm. Shifts in the absorption spectra were monitored after incubation times of 0, 15, 30, 60, and 120 min.

FA-Ara as a Substrate for Measurement of Esterase Activity

Determination of feruloyl esterase activity in commercial enzyme preparations. FA-Ara solution (1.38 mM) was prepared in 20 mM sodium acetate buffer (pH 5.0, 0.01% NaN_3). Reaction mixtures contained 125 μl of FA-Ara, 100 μg of enzyme, and acetate buffer to a final volume of 200 μl. Solid enzyme preparations (20–40 mg)

and liquid preparations (100 μl) were dissolved or suspended in 2 ml of 20 mM acetate buffer to provide a concentrated protein solution for the reaction mixtures. The enzyme substrate mixtures were incubated 24 h at 25°C. A 5-μl aliquot was removed, spotted on a TLC plate, dried for 15 min at 45°C, and developed in solvent D. Substrate and the hydrolyzed FA were visualized under short-wavelength uv (254 nm) and marked with a pencil. FA-Ara and FA were also visualized by development for 10 min in I_2 vapor. Authentic FA and FA-Ara were included as standards on each plate. Enzyme mixtures were incubated for another 24 h to determine if additional activity could be visualized by TLC.

Spectrophotometric assay method for feruloyl esterase activity. FA-Ara (1.38 mM) was added to universal buffer pH 8 that was diluted 10-fold with H_2O (final pH 8.0). An aliquot of the FA-Ara–buffer mixture (250 μl) was added to a small culture tube (10 × 75 mm) followed by 25 μl of a 100× dilution of pentosanase (NOVO). NOVO pentosanase was selected for these assays because it contained relatively high feruloyl esterase activity. The enzyme and substrate were immediately mixed, and a 40-μl sample removed and added to 460 μl of 200 mM Na_2CO_3 to inactivate the enzyme and raise the pH to 11.5. After mixing, the absorption spectrum was determined from 500 to 250 nm and this was taken as the zero time point. Subsamples of 40 μl were removed at 4, 10, 20, and 40 min and treated analogously.

For routine analysis of esterase activity, the substrate was prepared as described above. Enzyme activity in preparations was determined by mixing 250 μl of substrate with 25 μl of enzyme solution and incubating for 20 min at 25°C. A 40-μl subsample was removed and added to 460 μl of 200 mM Na_2CO_3, and the absorbance read at 375 nm. Blank controls consisted of the above mixtures without enzyme additions or with boiled enzyme preparations.

Direct monitoring of enzyme activity was accomplished for some samples by monitoring the change in absorption of the FA-Ara compound at pH 7. A 40-μl sample of FA-Ara stock solution was mixed with 435 μl of universal buffer pH 7 in a 1-ml cuvette. The absorption of the mixture was monitored for 4 min to give a background reading before 25 μl of enzyme solution was added to the cuvette and mixed. Hydrolysis of FA-Ara was monitored by reading the absorption every min for the next 20 min. The enzyme solution was diluted to one-half strength and the reaction sequence repeated. Each enzyme concentration was run in duplicate.

Measurement of esterase activity in plant extracts. Oat seedlings (*Avena sativa* "Garry") were grown in total darkness for 5 days at 25°C on sterilized cheese cloth. Two grams of coleoptiles was harvested, chopped into 2-mm segments, and loaded into a 7-ml tissue grinder (Corning) with 4 ml of 50 mM KH_2PO_4 buffer (pH 7.0) containing 20 mM EDTA and 2 mM DTE. Samples were maintained on ice during grinding (10 min). The total homogenate was transferred to 15-ml Corex centrifuge tubes and insoluble material pelleted by centrifuging at 8500*g* for 15 min. Three 1-ml aliquots of the supernatant were transferred to microfuge tubes (1.5 ml). One tube was capped and placed in a boiling water bath for 15 min. After heating, the sample was centrifuged for 1 min (Beckman Microfuge E) and the supernatant transferred to a clean microfuge tube. A 20-μl sample of FA-Ara stock solution (1.38 mM) was added to each microfuge tube (containing either plant extract or boiled control). After mixing, 5 μl was removed, spotted on TLC plates, and developed using solvent D. Samples were allowed to incubate for 20 h at 25°C in darkness before a second sample was removed from each tube and assayed by TLC. For HPLC analysis, samples were boiled for 15 min and centrifuged for 2 min in a microfuge. The supernatant was removed from each and placed in a glass test tube, frozen in liquid N_2, and lyophilized. The resulting solid was suspended in ethyl acetate and filtered. The filtrate was evaporated to dryness and dissolved in MeOH (100 μl) prior to injection.

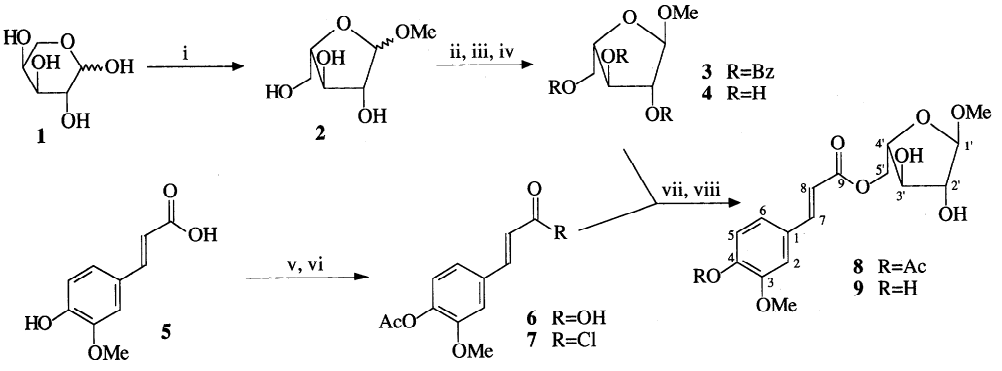
HPLC analysis of esterase activity against FA-Ara. HPLC separation of feruloyl esterase substrate (FA-Ara) and the hydrolytic product (FA) was carried out on a dual pump system (Gilson) using a C_{18} column (Whatman ODS Partisil-10, 10 μm, 0.5 × 25 cm) with a matching guard column. The mobile phase was MeOH:H_2O (1:1) and run at a flow rate of 1 ml/min. FA-Ara and FA were detected with a uv detector (Gilson uv detector Model 116) set at 325 nm (AUFS 0.150).

RESULTS AND DISCUSSION

Synthesis and Characterization of Methyl 5-O-trans-Feruloyl-α-L-arabinofuranoside

The synthesis of the FA-Ara (**9**) was straightforward as shown in the synthetic scheme (Fig. 1). L-Arabinose (**1**) was converted to a mixture of predominantly the methyl L-arabinofuranosides (**2**) by a standard Fischer glycosidation reaction. Monitoring the reaction by TLC ensured the maximum yield of the furanosides. It was possible to purify this mixture by flash chromatography without derivatization (30,33). However, benzoylation provided a material (**3**) which selectively crystallized from ethanol and was not hygroscopic. Debenzoylation of **3** to produce **4** (Fig. 1) was essentially quantitative, with losses occurring during crystallization. Use of the syrupy compound **4** (Fig. 1) in the subsequent coupling reaction proceeded without any difficulties as long as the material was dry.

The phenolic hydroxyl of ferulic acid (**5**) must be protected prior to formation of the acid chloride **7** to prevent polymerization. This was readily accomplished by acetylation; the acetyl group survived the acid chloride



(i) H_2SO_4/MeOH; (ii) BzCl/pyridine; (iii) selective crystallization; (iv) NaOMe; (v) Ac_2O/pyridine; (vi) $SOCl_2$; (vii) pyridine; (viii) piperidine

FIG. 1. Synthetic scheme for the production of FA-Ara.

step and could be quantitatively removed with piperidine. The coupling reaction exploited the slightly higher reactivity of the primary hydroxyl of **4** toward acylation. A slight deficit of **7** was used to minimize formation of di- and trisubstituted compounds.

The ^1H NMR spectrum of **9** exhibited an acylation shift of the C-5′ protons of the L-arabinofuranosyl moiety (H-5′a and H-5′b: **4**, 3.46 and 3.61 ppm; **9**, 4.27 and 4.40; respectively). Correlation of the hydroxyl protons at 4.64 and 4.44 ppm (these resonances disappear upon the addition of D_2O) with H-2′ and H-3′ in the COSY spectrum of **9** preclude feruloyl moiety attachment at these positions. The *trans* nature of the feruloyl double bond was indicated by the large $J_{7,8}$ coupling constant (15.9 Hz). The ^{13}C NMR data for the compounds investigated are shown in Table 1. The assigned resonances were based on two-dimensional COSY and C–H correlation NMR experiments, with long-range $^{13}C/^1H$ experiments used for assignment of the quaternary carbons. Figure 2 shows the long-range spectrum of **9** with superimposed schematic correlations obtained from the one-bond C–H correlation experiment. Of particular note is the correlation between C-9 and one of the H-5′ protons, confirming the esterification of C-5′ of the L-arabinofuranosyl moiety. The quaternary aromatic assignments were unambiguously made from the correlations (Fig. 2) of the 3-methoxyl protons to C-3 (3-bond), and of the 4-OH proton to C-4 (2-bond), C-3 (3-bond) and C-5 (3-bond) (29).

Analysis of uv spectra revealed that the absorption maximum of FA-Ara was distinct from that of FA over the range of solution pH values tested (Table 2) The ester linkage in FA-Ara shifted the absorption maximum to higher wavelengths compared to the maximum for FA. Over the pH range used the difference in absorption maximum varied from 17.5 to 65 nm. In the lower pH solutions (pH 5–8), FA contained two peaks (Table 2) that had nearly equal absorption intensities. Both compounds exhibited absorption maximum shifts to longer wavelengths (bathochromic shifts) as the pH increased. This was expected because electron transfer is facilitated in the anion, formed by ionization of the phenol at around pH 8–9 (34).

There was a transition stage that occurred for both compounds as the pH was increased. For FA-Ara it was at pH 8.0 and for FA it was pH 9.0. For both compounds, at the transition stage, the absorption maximum decreased in intensity as illustrated by a decrease in the

TABLE 1

^{13}C NMR Data (ppm) of Intermediates and Final Product of Synthesis

	Compounds[a]						
Carbon	**3**	**4**	**5**[b]	**6**[b]	**7**	**8**	**9**
1	—	—	125.9	133.3	132.9	134.1	127.4
2	—	—	111.3	111.9	113.4	112.3	111.2
3	—	—	148.0	151.2	152.9	152.6	148.7
4	—	—	149.2	140.9	144.1	142.6	150.1
5	—	—	115.7	123.2	124.4	124.0	116.0
6	—	—	122.9	121.4	123.9	122.2	124.1
7	—	—	144.7	143.4	151.5	145.0	146.1
8	—	—	115.8	119.6	122.9	118.8	115.5
9	—	—	168.2	167.7	166.2	166.9	167.4
OMe	—	—	55.8	56.0	56.5	56.3	56.3
C=O (Ac)	—	—	—	168.4	168.7	168.8	—
Me (Ac)	—	—	—	20.4	20.4	20.4	—
1′	107.6	110.3	—	—	—	110.3	110.3
2′	83.2	86.0	—	—	—	83.1	83.2
3′	78.9	78.8	—	—	—	79.2	79.3
4′	81.4	82.6	—	—	—	82.3	82.4
5′	64.4	62.7	—	—	—	65.0	64.8
OMe	54.9	54.7	—	—	—	55.0	55.0

[a] In acetone-d_6 referenced to the central solvent peak at 29.8 ppm. Compounds are as numbered in the synthetic scheme shown in Fig. 1.
[b] In DMSO-d_6 referenced to the central solvent peak at 39.5 ppm.

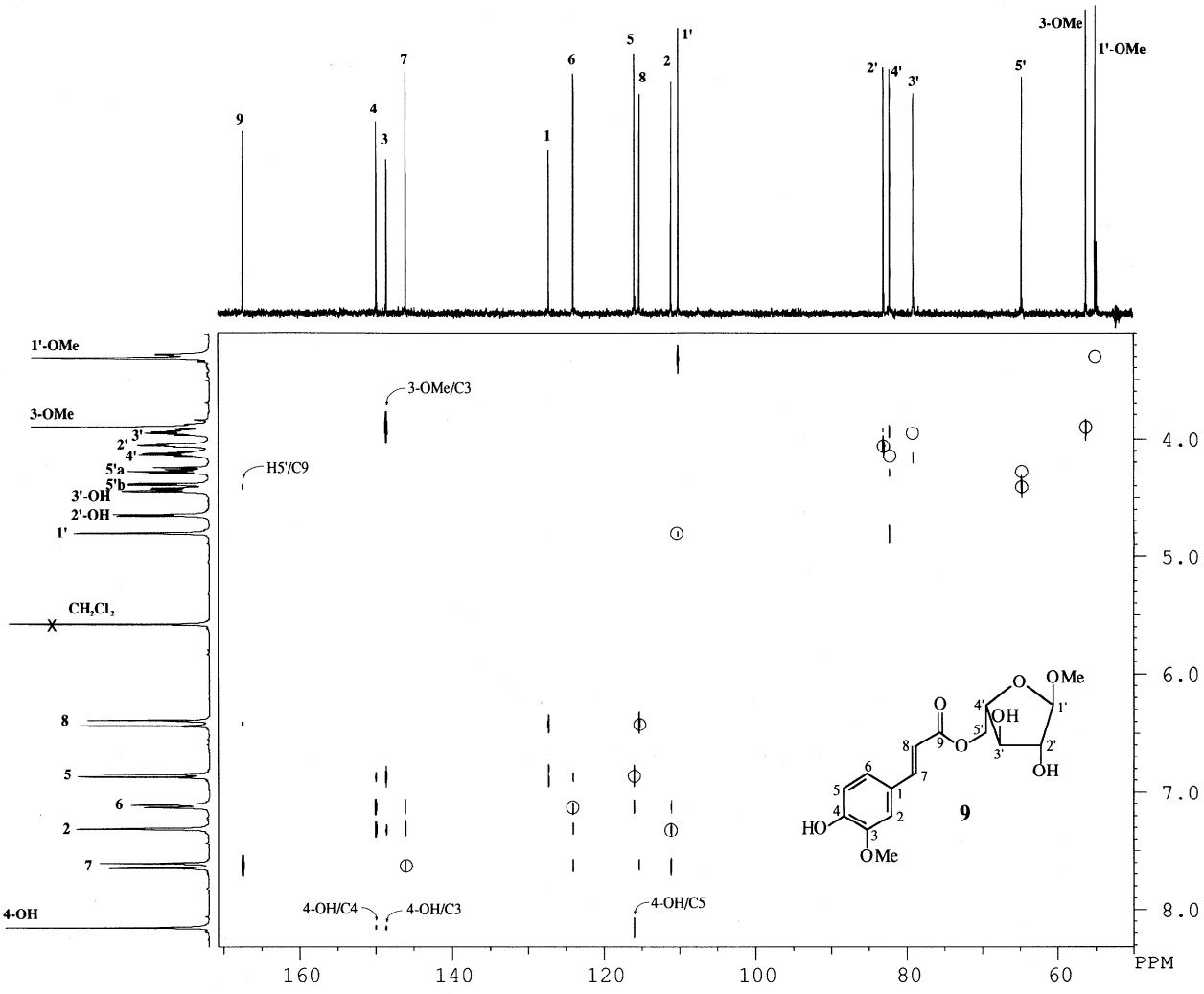


FIG. 2. Long-range ^{13}C–^{1}H correlation spectrum of **9.** Superimposed 1-bond correlation data is schematically indicated by open circles. See Materials and Methods for acquisition and processing details.

molar extinction coefficient (Table 2). The spectra became more complex with the formation of a second shoulder at 340 nm for FA and 370 nm for FA-Ara. The greatest difference in absorption maximum (65 nm) between the two compounds occurred at pH 9. At higher pH values each compound exhibited narrower, more defined maxima. The absorption spectra of FA-Ara and FA at pH 11 and higher were relatively narrow (Fig. 3) with absorption maxima that were well separated (35 nm). The intensity of the absorption also increased at this higher pH. This had the advantage of providing increased sensitivity for detection of the two compounds.

FA-Ara as a Substrate for Assessing Feruloyl Esterase Activity

TLC method. The TLC method for monitoring activity is flexible and does not require specialized equipment. The method is sensitive enough to detect 1.5 nmol of FA or FA-Ara. Since both compounds are visualized by either uv or I_2 treatment, TLC can be used to monitor the conversion of FA-Ara to FA. Both compounds are well resolved with R_f values of 0.70 (FA) and 0.52 (FA-Ara) in solvent D.

Several commercial enzyme preparations were tested with the FA-Ara compound to determine the presence of esterases capable of hydrolyzing feruloyl–arabinose–ester linkages. Of the preparations tested, half exhibited feruloyl esterase activity (Table 3). We did not attempt to further characterize the activity in positive samples since we were mainly interested in determining whether the preparations exhibited specific feruloyl esterase activity over extended incubation times. Such long-term incubations are frequently used when preparing partial cell wall digests (35). After 24 h those preparations exhibiting activity had completely degraded the FA-Ara to FA and methyl α-L-arabinofuranoside. Those prepara-

TABLE 2

Spectral Analysis of FA-Ara and FA: Summary of Absorption Maxima at Different Buffer pH Values and Calculated Molar Extinction Coefficients

Buffer pH	Wavelength of absorption maxima					
	FA-Ara			FA		
	1° peak	2° peak[a]	log ϵ^b	1° peak	2° peak	log ϵ^b
5	322.5	295.0	4.22	305.0	285.0	4.14
6	322.5	295.5	4.22	305.0	285.0	4.14
7	322.5	295.5	4.22	305.0	285.0	4.14
8	325.0	295.5	4.16	305.0	285.0	4.13
9	370.0	310.0	4.28	305.0	290.0	4.06
10	372.0	306.0	4.35	340.0	302.5	4.30
11	375.0	306.0	4.35	340.0	305.0	4.33
12	375.0	306.0	4.36	340.5	305.0	4.33

[a] The 2° peak was a shoulder on the 1° peak.
[b] log ϵ was based on the absorption maximum of the 1° peak.

TABLE 3

Determination of Feruloyl Esterase Activity in Commerical Enzyme Preparations

Enzyme Preparation	Source	Activity
Cellulysin	Calbiochem	—[a]
Novozym	NOVO	—
Celluclast	NOVO	—
Driselase	Sigma	—
Cellulase Type I	Sigma	++
Cellulase Type V	Sigma	—
Pectinex AR	NOVO	++
Pentosanase	NOVO	++
Pectinase	Worthington	++
Pectinase	Sigma	++

Note. Esterase activity was assessed by TLC after 24-h incubation at 25°C.
[a] Enzyme preparations that did not show activity after 24 h remained inactive after an additional 24-h incubation.

tions not exhibiting activity in the first 24 h did not show feruloyl esterase activity after an additional 24-h incubation. The TLC analysis method was relatively fast and easy, providing a simple assay that could be run on different enzyme preparations or used to evaluate different commercial lots of the same enzyme preparation. Because the synthetic substrate contains the same linkage (ferulic acid esterified to C-5 of an α-L-arabinofuranoside) as typically found in plant cell walls, activity against it would show conclusively which enzyme preparations would alter the native ferulic acid ester linkages to polysaccharides. FA-Ara also allows determination of whether esterases found in plants or ruminant microflora are capable of hydrolyzing the feruloyl ester linkage.

Monitoring esterase activity spectrophotometrically. Because of the difference in absorption maxima at all pH values, the hydrolytic conversion of FA-Ara to free FA could be monitored spectrophotometrically. Alkalinization of the enzyme solutions inactivates enzymes and increases sensitivity. A solution of 200 mM Na_2CO_3 was adequate to shift the pH to 11.5. The assay was performed by incubating the enzyme preparation with FA-Ara, removing 40 μl and adding it to 460 μl of Na_2CO_3, mixing, and reading the absorbance at 375 nm. Figure 4 illustrates the shift in absorbance spectrum as FA-Ara is hydrolyzed to FA and methyl α-L-arabinofuranoside. Monitoring the absorbance at 375 nm (340 nm for FA) allows a wide linear range over which activity can be measured. Monitoring the decrease in absorption as FA-Ara is hydrolyzed allows for a wider range of sensitivity and lower background reading than monitoring the increase in absorption due to the production of FA. A concern, however, is the saponification of FA-Ara at this higher pH. Although the compound will degrade at

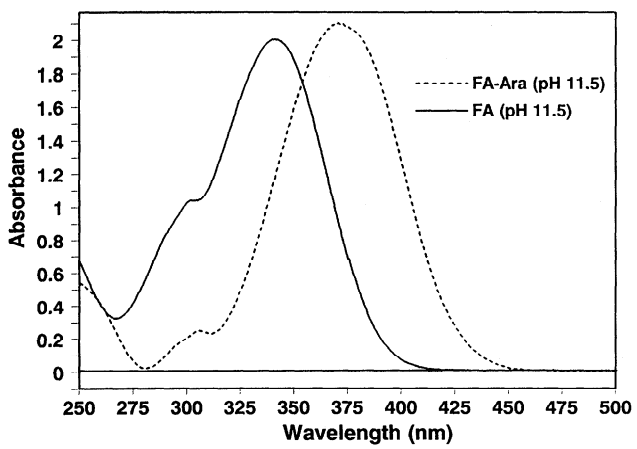


FIG. 3. Absorption spectrum of FA-Ara and FA at pH 11.5.

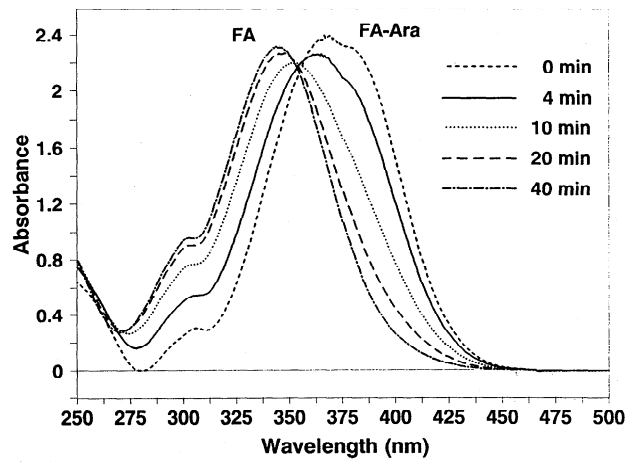


FIG. 4. Shift in the absorption maximum of FA-Ara as it is hydrolyzed to FA.

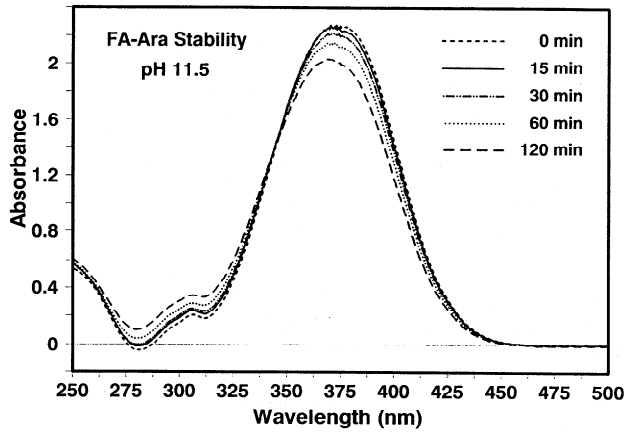


FIG. 5. Stability of FA-Ara in buffers made pH 11.5 by the addition of 200 mM Na_2CO_3.

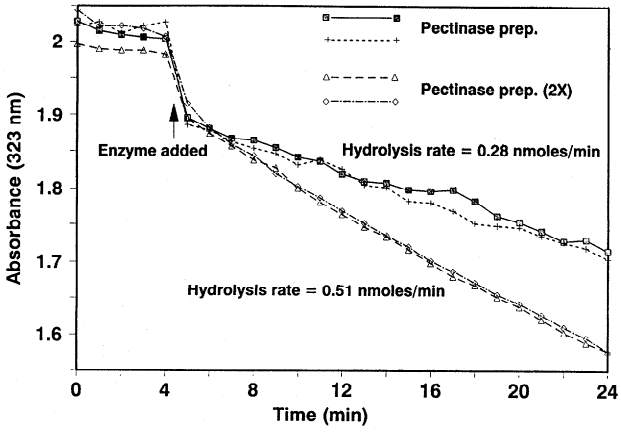


FIG. 7. Continuous spectrophotometric monitoring of feruloyl esterase activity in a commercial pectinase. Hydrolytic activity was calculated from the rate of change in absorbance at 323 nm.

pH 11.5, its hydrolysis is relatively slow, with losses of less than 10% over a 2-h period at 25°C (Fig. 5). It is therefore possible to set up several samples and read them within a relatively short time period without significant degradation.

Monitoring esterase activity by HPLC. Liquid chromatography has been used in previous studies for evaluating the enzymatic release of phenolic acids from plant cell walls (23,36,37). As can be seen in Fig. 6, HPLC can be used to monitor the cleavage of FA-Ara. Crude oat extracts contain at least one esterase that is capable of hydrolyzing the FA-Ara ester linkage. Lack of cleavage of the ester linkage by the boiled control is indicative of an enzyme-mediated reaction as opposed to buffer saponification. We found that the reaction could be monitored qualitatively by TLC, which is rapid and convenient. However, quantitation of substrate and products is not straightforward without specialized equipment. Using HPLC, it is possible to develop standard response curves for both FA-Ara and FA to quantify the amount of FA-Ara hydrolyzed and/or the amount of FA released. The use of a uv detector (325 nm), however, precludes the detection of L-arabinose; a refractive index detector would detect all reaction products, but at a significant loss in sensitivity.

Lyophilization of the total reaction mixture after boiling to denature proteins present in the sample allows the use of small amounts of substrate. Preliminary extraction with ethyl acetate solubilizes FA and FA-Ara with a minimum of contaminating compounds (particularly ionics). Resolubilization in MeOH allows for additional concentration of samples if necessary, and dissolves the sample in a solvent similar to the mobile phase of the C_{18} column.

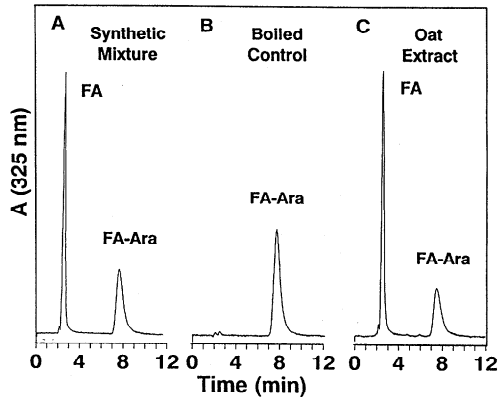


FIG. 6. HPLC separation of FA and FA-Ara on a C_{18} column. A, standard mixture of 0.36 μM FA-Ara and 0.75 μM FA. B, FA-Ara incubated with boiled enzyme extract from oat seedlings. C, same as B except with active enzyme extract.

Enzyme Kinetics

Enzyme kinetics for feruloyl esterases could be determined in small volumes within the spectrophotometer cuvette by continuously monitoring the decrease in the FA-Ara maximum or the increase in the FA maximum. Some care must be exercised here due to the overlap in the FA-Ara and FA spectra. However, there is sufficient difference to allow the development of a linear response with the proper selection of time intervals and protein concentration. The activity could be monitored at any pH within the range of 5 to 12. Figure 7 shows the activity of feruloyl esterase in a commercial pectinase from *Aspergillus* (Sigma) at two different concentrations. Data were collected directly from the spectrophotometer over a 25-min period. The advantage of this system was the ease with which one could monitor esterase activity. From the molar extinction coefficients of FA-Ara or FA at the pH used (Table 2) the molar reaction rates can be calculated. An acceptable range for FA-Ara detection at pH 8.0 would be 7 to 175 μM, whereas at pH 11.0 it would be 4 to 110 μM. Although only one reaction

could be monitored at a time, typically, reaction rates could be determined in a matter of minutes.

Summary

The synthesis of FA-Ara provides a specific substrate that effectively models the polysaccharide–feruloyl ester linkage that is frequently found in plant cell walls. Because FA-Ara is readily soluble in H_2O, solutions can be prepared in a wide range of concentrations without first solvating in an organic solvent. Assaying hydrolytic activity against FA-Ara can be accomplished by a range of methods including TLC, uv spectrophotometry, or HPLC. There is no need for secondary derivatization to follow enzyme reactions.

Using this substrate, it was possible to evaluate several commercial enzyme preparations for feruloyl esterase activity. Cellulysin, a cellulase preparation derived from *Trichoderma viride* cultures, and Driselase, a broad spectrum enzyme mixture from *Basidiomycetes*, are frequently used in cell wall digestion schemes for the isolation of wall components (5,35). Our results indicate that both preparations were free of feruloyl esterase activity. There were earlier reports that enzyme preparations from *T. viride* contained esterase activity capable of releasing ferulic acid from plant cell walls (3). Driselase on the other hand was reported to be free of feruloyl esterase activity (16). It was interesting to note that cellulase type V (Sigma), also derived from *T. viride*, did not contain feruloyl esterase activity, but cellulase type I derived from *Aspergillus* did. This would indicate that feruloyl esterase activity in enzyme preparations may be dependent upon the organism from which they were derived, and/or culture conditions used to produce specific enzyme mixtures.

Hydrolysis of FA-Ara by enzyme extracts from oat plants indicates the presence of feruloyl esterase activity in these plants. Whether this esterase is specific for feruloyl esters or has a wide substrate range is not known at this time nor is how widespread it is in the plant kingdom. It does indicate that, when isolating cell walls for the analysis of feruloyl esters, care should be taken to inactivate hydrolytic enzymes that may release ferulic acid.

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